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D-Amino Acids Enhance the Activity of Antimicrobials against Biofilms of Clinical Wound Isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Within wounds, microorganisms predominantly exist as biofilms. Biofilms are associated with chronic infections and represent a tremendous clinical challenge. As antibiotics are often ineffective against biofilms, use of dispersal agents as adjunctive, topical therapies for the treatment of wound infections involving biofilms has gained interest. We evaluated *in vitro* the dispersive activity of D-amino acids (D-AAs) on biofilms from clinical wound isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*; moreover, we determined whether combinations of D-AAs and antibiotics (clindamycin, cefazolin, oxacillin, rifampin, and vancomycin for *S. aureus* and amikacin, colistin, ciprofloxacin, imipenem, and ceftazidime for *P. aeruginosa*) enhance activity against biofilms. D-Met, D-Phe, and D-Trp at concentrations of ≥5 mM effectively dispersed preformed biofilms of *S. aureus* and *P. aeruginosa* clinical isolates, an effect that was enhanced when they were combined as an equimolar mixture (D-Met/D-Phe/D-Trp). When combined with D-AAs, the activity of rifampin was significantly enhanced against biofilms of clinical isolates of *S. aureus*, as indicated by a reduction in the minimum biofilm inhibitory concentration (MBIC) (from 32 to 8 μg/ml) and a >2-log reduction of viable biofilm bacteria compared to treatment with antibiotic alone. The addition of D-AAs was also observed to enhance the activity of colistin and ciprofloxacin against biofilms of *P. aeruginosa*, reducing the observed MBIC and the number of viable bacteria by >2 logs and 1 log at 64 and 32 μg/ml in contrast to antibiotics alone. These findings indicate that the biofilm dispersal activity of D-AAs may represent an effective strategy, in combination with antimicrobials, to release bacteria from biofilms, subsequently enhancing antimicrobial activity.

Chronic wounds are common in individuals with underlying medical conditions, such as diabetes mellitus, as well as in wounds resulting from traumatic injury, and significantly contribute to patient morbidity (1–3). A major factor contributing to the development of chronic wounds is colonization and subsequent infection by microorganisms. Recent studies evaluating the wound microbiota of chronic wounds of various etiologies have demonstrated that chronic wounds are often colonized by multiple bacterial species, of which *Staphylococcus* spp. and *Pseudomonas* spp. are two of the most commonly isolated organisms (4, 5). Within wounds, bacteria predominantly adopt a surface-attached mode of growth known as a biofilm. In brief, biofilms are an association of single or multiple microbial species surrounded by a self-produced, extracellular polymeric matrix, constituting a protected mode of growth (6–8). In contrast to their planktonic counterparts, biofilm-derived bacteria have a distinctive phenotype in regard to metabolic activity and gene expression, conferring an inherent resistance to antimicrobial agents as well as mechanisms of host clearance, making the treatment of biofilm-associated infections extremely difficult (9, 10).

The presence of bacterial biofilms within wounds is cited as a significant factor contributing to the chronicity and pathogenesis of wound infections (7, 11–13). For both *Staphylococcus aureus* and *Pseudomonas aeruginosa*, biofilm formation has been extensively documented *in vitro* and *in vivo* within chronic wounds (12, 14, 15). Importantly, the development and establishment of biofilms by both of these wound pathogens have been shown to directly impede wound healing and contribute to the development of chronic wounds (16–19). Given the importance of the biofilm phenotype in wound pathogenesis and the limitations of conventional antimicrobials against this phenotype, new strategies are needed for the treatment of chronic wounds.

Biofilm dispersal is a highly coordinated process, dependent on multiple factors, including cell density as well as responses to environmental cues, such as quorum-sensing signals and nutrient availability. To date, studies evaluating the late stages of biofilm growth and dispersal for a number of organisms, including *S. aureus* and *P. aeruginosa*, have identified multiple mechanisms that contribute this process (20–25). As a result, there have been tremendous research interest and focus of efforts in the identification of dispersive molecules that can be used to inhibit/disperse bacterial biofilms (20). Recently, for the soil bacterium *Bacillus subtilis*, the D-isoforms of various amino acids, including D-Leu, D-Met, D-Trp, and D-Tyr, were reported to have both inhibitory and dispersive activity against biofilms of *B. subtilis* (26, 27). In contrast to other biofilm dispersal agents that act to interfere with a single process essential for biofilm development, the dispersive

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TABLE 1 Characteristics of strains used in this study

Bacterial species and strains	Pulsed-field type	Phenotype[a]	Isolate source	Site of isolation
Pseudomonas aeruginosa				
SAMMC-604	1	MDR	Wound culture	Tissue deep
SAMMC-015	2	MDR	Blood	Blood
SAMMC-886	2	MDR	Wound culture	Tissue deep
SAMMC-418	18	MDR	Wound	Tissue deep
SAMMC-189	18	MDR	Blood	Blood
PAO1 (ATCC 15692)[b]	Unknown	Unknown	Wound	Unknown
Staphylococcus aureus				
SAMMC-700	USA 100	MRSA	Wound culture	Tissue deep
SAMMC-641	USA 200	MRSA	Wound culture	Tissue deep
SAMMC-474	USA 800	MRSA	Wound culture	Tissue deep
SAMMC-446	USA 300	MRSA	Wound culture	Tissue deep
SAMMC-240	USA 300	MRSA	Wound culture	Tissue deep
UAMS-1 (ATCC 43290)[c]	USA 200	MSSA	Wound culture	Bone

[a] A multidrug-resistant (MDR) organism was defined as an organism resistant to antimicrobials in ≥3 classes of antimicrobial agents (penicillins/cephalosporins, carbapenems, aminoglycosides, and quinolones), not including tetracyclines or colistin. MRSA, methicillin-resistant *Staphylococcus aureus*.
[b] *Pseudomonas aeruginosa* strain PAO1 is a well-characterized and commonly used wound isolate (34, 35).
[c] *Staphylococcus aureus* strain UAMS-1 is a methicillin-susceptible and well-characterized osteomyelitis isolate of *S. aureus* (32, 33).

activities of D-amino acids (D-AAs) have been attributed to multiple mechanisms, including (i) inhibition of growth and expression of genes involved in biofilm matrix production (28) as well as (ii) diminished surface expression of fibers involved in biofilm formation, resulting from incorporation of D-AAs into the bacterial cell wall (26). In addition to their activity against *B. subtilis* biofilms, D-AAs have also been shown to have dispersive activity against biofilms of *S. aureus* and *P. aeruginosa in vitro* (27, 29) and biofilms of *S. aureus in vivo* when incorporated into a modified bone graft (30).

Given these observations, we hypothesized that combining dispersal agents with antimicrobials may be an effective therapeutic strategy for biofilms, functionally restoring susceptibility of biofilms to antimicrobials through the release of bacteria from the biofilm. To explore this hypothesis, we evaluated the dispersal activity of D-AAs on biofilms of clinical wound isolates of *S. aureus* and *P. aeruginosa* and investigated whether combining D-AAs with various classes of antibiotics enhances the activity against biofilm-producing bacteria *in vitro*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The clinical isolates utilized in this study were selected from a strain collection at the Brooke Army Medical Center/San Antonio Military Medical Center (BAMC/SAMMC) (Fort Sam Houston, TX, USA) and previously characterized for biofilm formation (Table 1) (31). Bacterial isolates from this strain collection were collected from patients in the course of routine clinical care not related to research. *S. aureus* strain UAMS-1 (ATCC 25943) is a methicillin-susceptible osteomyelitis isolate (32, 33). *P. aeruginosa* strain PAO1 is a well characterized wound isolate widely used as a laboratory strain (34, 35). For planktonic growth, clinical strains of *S. aureus* and *P. aeruginosa* were cultured in tryptic soy broth (TSB) and Luria-Bertani broth (LB), respectively, at 37°C. Bacteria were subcultured on blood agar plates (Remel, Lenexa, KS, USA) overnight at 37°C.

Antibiotics and D-amino acids. For *S. aureus* clinical strains the following antibiotics and concentrations were used; clindamycin (CLI) (0.25 to 1,024 μg/ml), cefazolin (CFZ) (0.25 to 1,024 μg/ml), oxacillin (OXA) (0.125 to 1,024 μg/ml), vancomycin (VANC) (0.125 to 1,024 μg/ml), and rifampin (RIF) (0.125 to 1,024 μg/ml). For *P. aeruginosa*, amikacin (AMK) (0.5 to 1,024 μg/ml), colistin (CST) (0.25 to 1,024 μg/ml), ciprofloxacin (CIP) (0.125 to 1,024 μg/ml), imipenem (IPM) (0.25 to 1,024 μg/ml), and ceftazidime (CAZ) (0.25 to 1,024 μg/ml) were used. Antibiotics were purchased from Sigma-Aldrich (St. Louis, MO). Stock solutions were prepared as recommended and diluted to the appropriate concentrations in cation-adjusted Mueller-Hinton broth (MHB-II). Quality control strains included *S. aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853. D-amino acids were purchased from Sigma-Aldrich and prepared as concentrated stock solutions in water or 1.0 N HCl, followed by filter sterilization. From the prepared stock solutions, D-AAs were diluted into MHB-II to a final concentration of 50 mM and neutralized when necessary with NaOH (1 M) (pH 7 to 7.4). All subsequent working concentrations of D-AAs were prepared by diluting the neutralized 50 mM stock into MHB to yield final working concentrations.

Biofilm formation in 96-well plates and biofilm dispersal assays. Biofilm formation was performed under static conditions for 24 h in polystyrene 96-well plates (Corning, Inc., Corning, NY) as described previously (31). Briefly, following overnight incubation, medium was removed from individual wells and washed with 1× phosphate-buffered saline (PBS), and 200 μl of media without or supplemented with D-AAs at the designated concentrations in MHB-II was added to each well for an additional 24 h. Following overnight exposure, cells were washed as above and biofilm biomass was determined by measuring the optical density at 570 nm (OD_{570}) of crystal violet solubilized in ethanol. Experimental assays were performed in triplicate.

Confocal scanning laser microscopy. *S. aureus* and *P. aeruginosa* biofilms were visualized using a FluoView confocal laser-scanning microscope (Olympus, Pittsburgh, PA). Biofilms were grown as described above in 8-well glass chamber slides (36). Biofilms were stained with dual combinations of biofilm ruby matrix stain and biofilm cell stain (Molecular Probes, Eugene, OR) to visualize the extracellular polymeric matrix and bacterial cells, respectively, according to the manufacturer's instructions. Confocal scanning laser microscopy (CLSM) images were acquired at ×20 magnification using a HeNe-G laser at 543 nm for the matrix stain and an argon laser (488 nm) for bacteria. Image analysis and z-stacks were acquired using the Olympus FluoView software. Images were taken from three distinct regions on the slide and representative images were selected for each treatment group.

Antibiotic susceptibility of planktonic bacteria. Antibiotic susceptibilities of selected clinical strains were evaluated by determining the MIC as recommended by the Clinical and Laboratory Standards Institute (37). Test performance for antimicrobial agents was monitored using *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 as control strains. For each

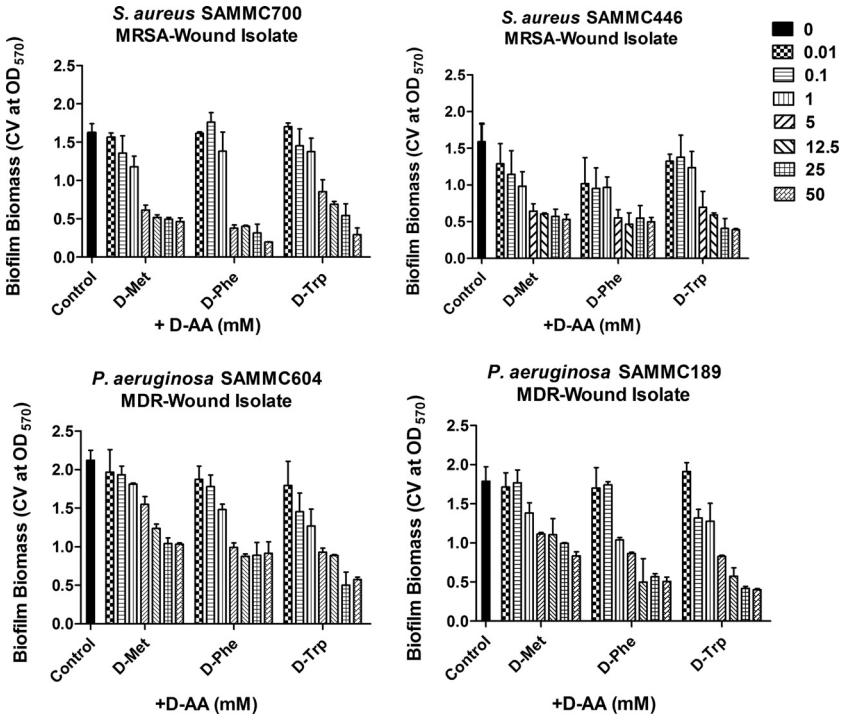

FIG 1 Dose-dependent effects of D-AAs against *S. aureus* and *P. aeruginosa* biofilms. Screening of dispersive activity of individual D-amino acids, D-methionine (D-Met), D-phenylalanine (D-Phe), and D-tryptophan (D-Trp) (at concentrations ranging from 0.01 to 50 mM) against biofilms of two clinical methicillin-resistant *S. aureus* (MRSA) isolates (top panels) and two multidrug-resistant (MDR) isolates of *P. aeruginosa* (bottom panels). Biofilm dispersal was assessed by measuring the absorbance of solubilized crystal violet from stained biofilms following overnight treatment with D-AAs at 570 nm. *, $P < 0.05$ versus untreated control. CV, crystal violet.

antibiotic tested, the MIC of planktonic organisms was determined in the presence or absence of the D-AA mixture (1:1:1 D-Met/D-Phe/D-Trp). Antimicrobial susceptibility assays were performed in duplicate.

Antibiotic susceptibility of biofilm bacteria and determination of the minimal biofilm inhibitory concentration. The method for determination of the MBIC for biofilm bacteria following antimicrobial treatment was adapted from previously described studies using modified minimum biofilm eradication concentration high-throughput (MBEC-HTP) assay plates (Innovotech, Canada) for biofilm antimicrobial susceptibility testing (38, 39). Briefly, bacteria were inoculated into wells containing either TSB or LB, for *S. aureus* and *P. aeruginosa*, respectively, covered with a lid containing pegs for the attachment of the bacteria, and incubated at 37°C for 48 h with agitation. Following incubation, plate lids containing the pegs with the attached biofilms were washed with 1× PBS and submerged in 2-fold serial dilutions of antibiotics diluted in MHB-II in 96-well plates (i.e., challenge plate) alone or in combination with the D-AA mixture (1:1:1 D-Met/D-Phe/D-Trp) overnight at 37°C. Plate lids were then removed, washed, and transferred to a new 96-well recovery plate with new culture media for determining the MBIC. The MBIC was defined as the lowest concentration of antibiotics at which no visible growth was observed after 6 h of recovery. Viability of bacteria on pegs immediately following antimicrobial exposure was determined by enumerating serial dilutions on blood agar plates (Remel, Lenexa, KS) following removal of bacteria by sonication. Antimicrobial susceptibility assays were performed in triplicate.

Statistical analysis. Multigroup comparisons were performed using a one-way ANOVA followed by a Dunnett's *post hoc* test for comparisons between test and control groups using GraphPad Prism version 5. Differences were considered to be statistically significant at *P* values of <0.05.

RESULTS

Dose-dependent effect of D-amino acids on *S. aureus* and *P. aeruginosa* biofilms. To evaluate the potential clinical application of D-AAs, we tested whether D-AAs effectively dispersed preformed biofilms of clinical isolates of *S. aureus* and *P. aeruginosa*. Prescreening of eight individual D-AAs identified three (D-Met, D-Phe, and D-Trp) that had potent activity at dispersing preformed biofilms of representative wound isolates of both *S. aureus* and *P. aeruginosa* (Fig. 1). In contrast, the other D-AAs tested (D-Ala, D-Ile, D-Leu, D-Tyr, and D-Val) had variable-to-minimal dispersive activity (data not shown). Dispersal activities of D-Met, D-Phe, and D-Trp were significant at concentrations of ≥5 mM. Of note, the pH of all tested concentrations of D-AAs diluted in MHB-II was neutral, indicating that the observed activity was independent of an effect from the pH of the solution. D-Met, D-Phe, and D-Trp were all observed to be effective against *S. aureus* biofilms (Fig. 1, top panels), whereas for *P. aeruginosa*, D-Trp and D-Phe had greater dispersive activity than D-Met (Fig. 1, bottom panels). Based on the initial screening of D-AA dispersal activity, 5 mM was chosen as the concentration for use in all subsequent *in vitro* assays.

D-Amino acids disperse biofilms of clinical wound isolates of *S. aureus* and *P. aeruginosa*. When tested against biofilms of genetically diverse clinical isolates of *S. aureus* ($n = 5$) and *P. aeruginosa* ($n = 5$), D-Met, D-Phe, and D-Trp at 5 mM were also observed to have significant dispersal activity, as indicated by the reductions in biofilm biomass as determined by crystal violet assay (Fig. 2A and B). As expected, the activity of D-AAs against biofilms

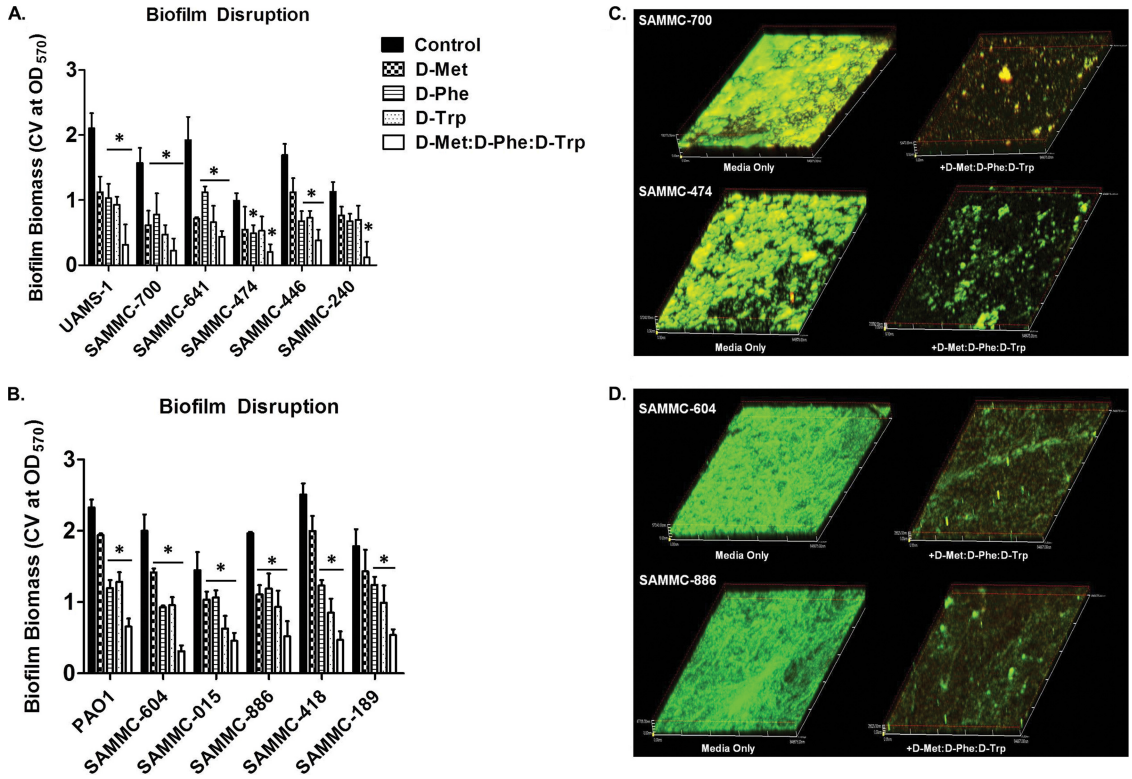

FIG 2 Biofilm dispersive activity of D-AAs on clinical wound isolates. Activity of D-AAs D-Met, D-Phe, and D-Trp individually at 5 mM and as an equimolar mixture (1:1:1 D-Met/D-Phe/D-Trp) against biofilms of methicillin-resistant (SAMMC-700, SAMMC-641, SAMMC-474, SAMMC-446 and SAMMC-240) and methicillin-susceptible (UAMS-1) *S. aureus* isolates (A) and multidrug-resistant (SAMMC-604, SAMMC-015, SAMMC-886, SAMMC-418, and SAMMC-189) strains of *P. aeruginosa* (B). Biofilm dispersal was assessed by measuring the absorbance of solubilized crystal violet from stained biofilms following treatment with D-AA at 570 nm. *, $P < 0.05$ versus untreated control. Representative CLSM images of biofilms of (C) *S. aureus* and (D) *P. aeruginosa* clinical isolates treated with an equimolar mixture of D-AAs for 24 h. Biofilms were stained with dual combinations of biofilm ruby matrix stain (red) and biofilm cell stain (green) and images were taken at a magnification of ×20.

was partly strain dependent, in particular for isolates of *P. aeruginosa*, although for each strain tested more than one of the D-AAs reduced biofilm to >50% of the untreated control. Notably, when combined as an equimolar mixture (1:1:1 D-Met/D-Phe/D-Trp), biofilm dispersal activity was enhanced compared to the exposure to individual D-AAs (Fig. 2A and B). Consistent with these results, confocal microscopy analysis of biofilms treated with the D-AA mixture demonstrated a significant reduction in biofilm biomass compared to that of untreated controls (Fig. 2C and D).

Importantly, D-AAs alone or in combination as a mixture had no significant effect on the growth of *S. aureus* and *P. aeruginosa* cells, indicating that biofilm dispersal was not the result of growth inhibition (see Fig. S1 in the supplemental material).

D-Amino acids enhance the activity of antimicrobials against biofilm bacteria but not planktonic bacteria. To determine if D-AAs could enhance the effect of conventional antimicrobials against bacteria within biofilms, antimicrobial susceptibility assays with and without the D-AA mixture (1:1:1 D-Met/D-Phe/D-Trp) were initially performed on both the planktonic (i.e., grown in liquid culture) and biofilm phenotype of two strains of *S. aureus* (UAMS-1 and SAMMC-700) and *P. aeruginosa* (SAMMC-418 and SAMMC-189). The MICs of antimicrobials for planktonic *S. aureus* strains (UAMS-1 and SAMMC-700, respectively) were determined for clindamycin (0.5 μg/ml), cefazolin (0.5 μg/ml and 16 μg/ml), oxacillin (1 μg/ml and 8 μg/ml), rifampin (0.5 μg/ml), and vancomycin (0.5 μg/ml and 1 μg/ml) (Table 2). Similarly, the MICs for the *P. aeruginosa* strains, SAMMC-418 and SAMMC-189, respectively, were determined for amikacin (16 μg/ml), colistin (1 μg/ml), ciprofloxacin (4 μg/ml; 8 μg/ml), imipenem (128 μg/ml and 64 μg/ml), and ceftazidime (16 μg/ml and 2 μg/ml) (Table 3). Notably, the combination of the D-AA mixture with antibiotics did not alter the susceptibilities of planktonic bacteria.

As anticipated, the majority of antimicrobial agents tested against biofilms of *S. aureus*, including clindamycin, cefazolin, oxacillin, and vancomycin, were ineffective at the tested concentrations against the biofilm phenotype, with observed MBICs exceeding the antimicrobial test range in most instances (Table 2). In contrast, rifampin was active against the biofilms of *S. aureus* at 32 μg/ml and 64 μg/ml for UAMS-1 and SAMMC-700, respectively. Interestingly, exposure to antimicrobials in combination with the D-AA mixture enhanced the activity of rifampin, clindamycin, and vancomycin against the biofilm phenotype (Table 2). Combined exposure resulted in reductions of the observed MBICs of 4- and 8-fold (2 and 3 2-fold dilutions) for rifampin, 6- and 4-fold (3 and 2 2-fold dilutions) for clindamycin, and 2- to 4-fold (2 2-fold dilutions) for vancomycin against *S. aureus* UAMS-1 and SAMMC-700, respectively. Notably, exposure of biofilms of other clinical isolates of *S. aureus* to combined treatments at the experimentally determined MBICs above for clindamycin, vancomycin, and rifampin (64 μg/ml for clindamycin and vancomycin and

TABLE 2 Minimal inhibitory concentration and minimal biofilm inhibition concentration of antibiotics alone or in combination with D-amino acids

Antimicrobial agent[a]	Class	Planktonic MIC[b] (μg/ml) for:		Biofilm MBIC[b] (μg/ml) for:	
		S. aureus UAMS-1	*S. aureus* SAMMC-700	*S. aureus* UAMS-1	*S. aureus* SAMMC-700
CLI	Lincosamide	0.5	0.5	256	512
CLI + D-AA		0.25	0.5	32	128
CFZ	Cephem	0.5	16	512	1,024
CFZ + D-AA		0.5	16	512	1,024
OXA	Penicillin	1	8	256	>1,024
OXA + D-AA		1	8	256	>1,024
RIF	Ansamycin	0.5	0.5	32	64
RIF + D-AA		0.5	0.5	8	8
VAN	Glycopeptide	0.5	1	256	512
VAN + D-AA		0.5	1	128	128

[a] CLI, clindamycin; CFZ, cefazolin; OXA, oxacillin; RIF, rifampin; VAN, vancomycin.
[b] MIC and minimum biofilm inhibition concentration (MBIC) of the antibiotic (μg/ml) alone or in combination with an equimolar mixture of D-Phe/D-Trp/D-Met (5 mM).

8 μg/ml rifampin) also resulted in a greater reduction of viable bacteria from biofilms than treatment with antimicrobials alone (Fig. 3). Exposure of biofilms of genetically distinct staphylococcal isolates to combined treatments reduced viable bacterial counts between 1.5 and 2 logs for clindamycin and vancomycin, whereas combination treatment resulted in a >2-log reduction for rifampin, compared to antimicrobial treatment alone.

Similarly, for *P. aeruginosa*, treatment of the biofilms with the selected panel of antimicrobials alone at the test ranges was ineffective against the biofilms (Table 3). However, as observed with *S. aureus*, the addition of the D-AAs enhanced the activity of colistin and ciprofloxacin against multiple clinical strains of *P. aeruginosa*, reducing the observed MBICs (Table 3) and bacterial viability (Fig. 4A and B) compared to antimicrobials alone.

DISCUSSION

Wound infections constitute a major burden of disease, with multiple factors complicating antimicrobial therapy, including antimicrobial resistance and biofilm formation. Biofilm formation is considered to be a significant pathogenic attribute for both invasive and opportunistic pathogens, such as *S. aureus* and *P. aeruginosa*. The importance of biofilms to clinical disease is illustrated by estimates indicating that more than 80% of all bacterial infections, in particular chronic infections, may involve a biofilm etiology (7, 8, 40). Although clinical evidence linking biofilms to recurrent or persistent clinical wound infections is scarce, we have recently observed biofilm formation in 61.4% of bacterial isolates of multiple bacterial species associated with clinical wound infections, demonstrating a higher degree of biofilm formation among tissue, bone, and respiratory isolates (31). Furthermore, we have also shown through a clinical case-control study that *in vitro* biofilm formation by infecting pathogens carries an odds ratio of 29.5 for persistent wound infection (41). Importantly, and as a result of growth within a biofilm, conventional antibiotics are often ineffective against biofilms, which require in most cases 10- to 1,000-fold-higher concentrations than their planktonic counterparts. Consequently, the biofilm phase of growth can further complicate therapy by conferring a high degree of innate resistance to antimicrobials, even those to which the causative bacteria are susceptible as planktonic organisms (9, 10, 42). Given the importance of the biofilms to disease and the limitation of conventional antimicrobials against this phenotype, herein we assessed whether the use of D-AAs, a biofilm dispersive agent, could enhance the activity of antimicrobials against biofilms.

Only recently have compounds and strategies been reported which specifically target biofilms, including D-AAs, promoting disassembly that may be used in combination with antimicrobials to enhance activity against biofilms (43, 44). The D-isoforms of amino acids represent one such example, and they have been pre-

TABLE 3 MIC and minimal biofilm inhibition concentration of antibiotics alone or in combination with D-amino acids

Antimicrobial agent[a]	Class	Planktonic MIC[b] (μg/ml) for:		Biofilm MBIC[b] (μg/ml) for:	
		SAMMC-189	SAMMC-418	SAMMC-189	SAMMC-418
AMK	Aminoglycoside	16	16	512	>1,024
AMK + D-AA		8	16	512	>1,024
CS	Lipopeptide	1	1	256	256
CS + D-AA		1	1	32	64
CIP	Fluoroquinolone	4	8	128	256
CIP + D-AA		4	8	32	32
IMI	Carbapenem	128	64	256	256
IMI + D-AA		128	64	256	128
TAZ	Cephem	16	2	512	>1,024
TAZ + D-AA		8	2	512	512

[a] AMK, amikacin; CS, colistin; CIP, ciprofloxacin; IMI, imipenem; TAZ, ceftazidime.
[b] MIC and minimum biofilm inhibition concentration (MBIC) of the antibiotic (μg/ml) alone or in combination with an equimolar mixture of D-Phe/D-Trp/D-Met (5 mM).

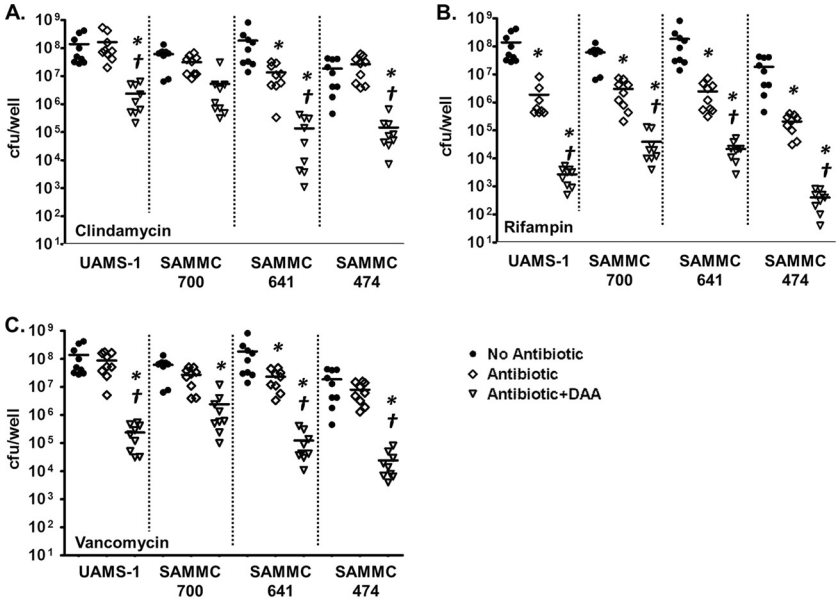

FIG 3 Effects of combinations of D-AAs and antibiotics against biofilms of clinical isolates of *S. aureus*. Biofilms of clinical methicillin-resistant (SAMMC-700, SAMMC-641, SAMMC-474) and methicillin-susceptible (UAMS-1) *S. aureus* were developed on pegs of MBEC-HTP plates (Innovotech) for 24 h, followed by exposure to clindamycin (64 μg/ml) (A), rifampin (8 μg/ml) (B), and vancomycin (64 μg/ml) (C) in the absence (◇) or presence of the D-AA mixture (1:1:1 D-Met/D-Phe/D-Trp) (▽) for 24 h. Viable bacteria from biofilms were determined by plating serial dilutions, following removal of adherent bacteria by sonication. Values are expressed as $\log_{10}$ (CFU/well). *, $P < 0.05$ versus control; †, $P < 0.05$ versus antibiotic alone.

viously shown to have dispersal activity against biofilms of *S. aureus* and *P. aeruginosa* (27, 45). Consistent with these studies, we observed that D-AAs, including D-Met, D-Phe, and D-Trp, when used individually, and to a greater extent as an equimolar combination, had significant dispersive activity on biofilms of multiple, genetically distinct clinical isolates at concentrations of ≥5 mM. This observed activity of individual D-AAs is consistent with previous reports indicating a range of activities for D-Met, D-Phe, and D-Trp of between 2 and 5 mM and 10 mM against biofilms of *S. aureus* and *P. aeruginosa*, respectively (27, 29, 45). Importantly, and in contrast to previous studies with *B. subtilis*, significant effects on either cell viability or bacterial growth were not observed at the effective concentrations (28). Collectively, these initial screening studies demonstrate that the dispersive activity of D-AAs functions in a strain-independent manner against common wound pathogens, such as *S. aureus* and *P. aeruginosa*, and are suggestive of the potential use in applications for infectious complications due to these agents.

Although the addition of D-AAs did not enhance the activity of antimicrobials against planktonic cells, the addition of D-AAs was observed to enhance the activity of several antibiotic classes against biofilms of genetically distinct isolates of methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant (MDR) *P. aeruginosa*. The greatest enhancement of antimicrobial activity by D-AAs was observed with rifampin (against *S. aureus*) and ciprofloxacin (against *P. aeruginosa*).

For rifampin and ciprofloxacin, combinations with D-AA demonstrated near-bactericidal activity, with 2- to 3-log CFU decreases compared to each agent alone. Additionally, we also observed 1- to 2-log CFU reductions with vancomycin, clindamycin, and colistin when combined with D-AAs compared to each agent alone. Notably, we did not observe potentiation of activity of oxacillin or cefazolin against methicillin-susceptible strains of *S. aureus* (strain UAMS-1), nor of amikacin, imipenem, or ceftazidime against *P. aeruginosa* when combined with D-AAs. In line with our findings, previous studies have demonstrated that the activity of various antimicrobial agents can be significantly enhanced by the addition of the dispersal agents, such as quorum-sensing inhibitors. In one study, the use of a quorum-sensing analog significantly enhanced the activity of several antibiotics, including tobramycin, against *P. aeruginosa* biofilms, as well as enhanced clearance in a foreign-body infection model (46). Similar results were also observed in studies examining the use of quorum-sensing inhibitors (QSI) against biofilms of *P. aeruginosa*, *Burkholderia cepacia*, and *S. aureus*, whereby the combined use of an antibiotic and a QSI resulted in increased killing compared to antibiotic alone (47). For D-AAs, as well as the quorum-sensing inhibitors, the mechanisms through which the combinations with these agents enhanced the activity of antimicrobials have not been fully addressed. In contrast to QSI inhibitors, which may modulate metabolic activity of bacteria as signaling occurs through two-component signaling mechanisms, D-AAs may have augmented the activity of antimicrobials against biofilms in part following dispersal of bacteria from biofilms. However, we cannot exclude the possibility of other effects of D-AAs, such as changes in protein expression, cell wall integrity, and metabolic status, which may also contribute to observed enhanced susceptibility *in vitro*.

The explanation for potentiation by D-AAs of some agents, but not others, against established biofilms is not readily apparent and requires further examination. Biofilm penetration on the basis of hydrophobicity does not appear to be the explanation for the inactivity of some agents, as log *P* values describing the degree of hydrophobic/hydrophilic partitioning ranged from −7.9 (amikacin) to 2.4 (oxacillin) versus −3.3 (colistin) to 4 (rifampin). Furthermore, the remaining agents found to be inactive in our study target cell wall penicillin-binding proteins and may be less active

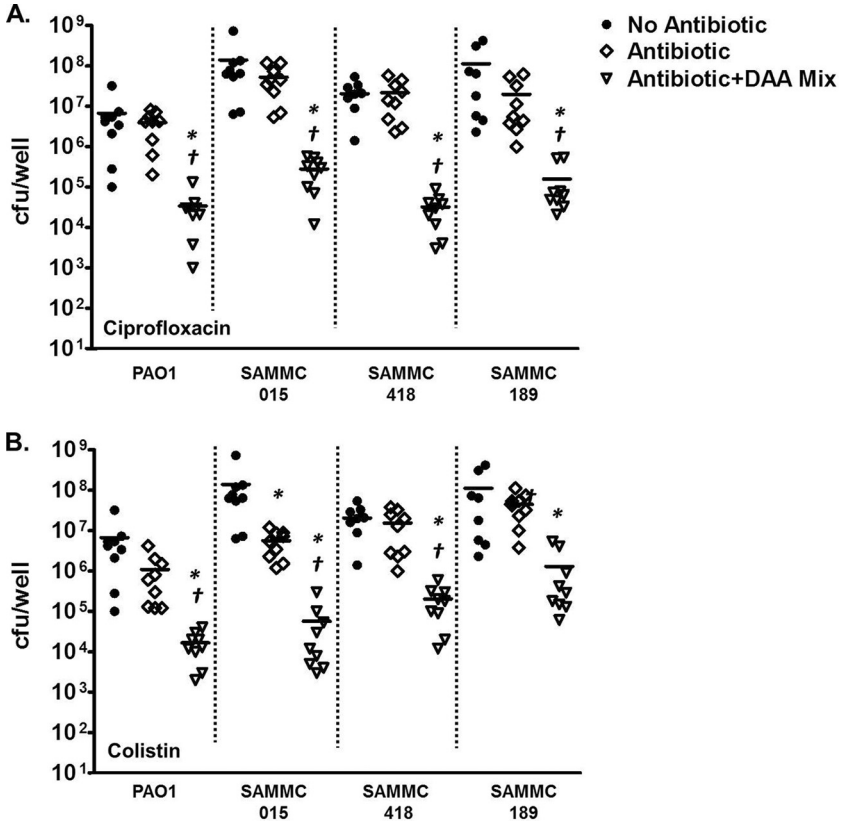

FIG 4 Effect of combinations of D-AAs and antibiotics against biofilms of clinical isolates of *P. aeruginosa*. Biofilms of multidrug-resistant clinical strains (SAMMC-015, SAMMC-418, and SAMMC-189) and a laboratory strain (PAO1) of *P. aeruginosa* were developed on pegs of MBEC-HTP plates (Innovotech) for 24 h, followed by exposure to ciprofloxacin (32 μg/ml) (A) or colistin (64 μg/ml) (B) in the absence (◇) or presence of the D-AA mixture (1:1:1 D-Met/D-Phe/D-Trp) (▽) for 24 h. Viable bacteria from biofilms were determined by plating serial dilutions, following removal of adherent bacteria by sonication. Values are expressed as $\log_{10}$ (CFU/well). *, $P < 0.05$ versus control; †, $P < 0.05$ versus antibiotic alone.

due to decreased bacterial replication. However, vancomycin and colistin, which were potentiated in combination with D-AA, also target bacterial cell wall constituents. Future studies are warranted to fully characterize these phenomena.

Our observation that these primary antimicrobial therapies can be enhanced to a bactericidal level of activity against biofilms by addition of D-AAs introduces the possibility that local delivery of antibiotics combined with D-AAs might be used to effectively reduce infections with little systemic toxicity. The concentrations of antimicrobials tested (selected as the MBIC for each agent) in combination with D-AAs, although higher than what are safely achievable by systemic administration, can potentially be reached by local antibiotic delivery within an infected wound. However, the local elution of antibiotics at high concentrations raises concerns for tissue toxicity and impaired wound healing (48). However, since antimicrobial concentrations required for biofilm eradication may be reduced by codelivery with a dispersal agent, toxicity can potentially be mitigated with their inclusion. In support of this concept, recently we have demonstrated the potential benefits of local delivery of antibiofilm agents *in vivo*, utilizing D-AA elution from an impregnated polyurethane scaffold implanted in a contaminated rat segmental bone defect model, which prevented *S. aureus* adherence and reduced microbial burden within the bone and on the graft (30). Thus, a safe and effective local delivery vehicle for the antibiofilm treatment of infected wounds might be possible by including both dispersal agents and antimicrobials which elute at subtoxic concentrations.

Importantly, we observed antibiofilm activity for these agents in the setting of very high inoculums (~10^8 CFU), which approximates the bacterial burden in an abscess or undebrided wounds at which the risk for development of resistance is increased (49). The recent observation of a "critical colonization" contamination threshold (~10^5 CFU/g tissue) associated with local and systemic elevations in inflammatory cytokines (50), which have been linked to wound dehiscence (51, 52), suggests that postdebridement adjunctive use of dispersal agents such as D-AAs might shorten the required duration of antimicrobial therapy for these difficult infections, translating into substantial cost savings and limiting complications of therapy. Whether the use of dispersal agents could eliminate the need for a second antimicrobial agent to protect against resistance requires further investigation.

In conclusion, we have demonstrated *in vitro* enhancement of antibiofilm activity to near-bactericidal levels, resulting from biofilm dispersal by D-AAs. Biofilm dispersal by individual amino acids was dose dependent, and amino acids in combination potentiated the antibiofilm activity of multiple antimicrobial classes against genotypically diverse isolates of *S. aureus* and *P. aeruginosa*, including several agents recommended as first-line treatments for osteomyelitis and prosthetic joint infections. Additional studies should confirm these findings *in vivo* and explore potential

delivery devices which could be used for the clinical treatment of osteoarticular infections mediated by bacterial biofilms.

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